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Responses of intertidal bacterial biofilm communities to increasing pCO_2 in a naturally acidified system

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Abstract

The effects of ocean acidification on ecosystems remain poorly understood, because it is difficult to simulate the effects of elevated CO_2 levels on entire marine communities in controlled laboratory conditions. Natural systems such as CO_2 seeps that are enriched in CO_2 are being used to help understand the long-term effects of ocean acidification *in situ*. Here, we compared biofilm bacterial community composition on cobbles/boulders and bedrock along a CO_2 gradient in the NW Pacific. Samples sequenced for 16S rRNA showed that different bacterial communities were associated with zones of different seawater pCO_2, and were also distinct between the two rocky habitat types. In both habitats, there was increased bacterial diversity in the biofilm communities in acidified conditions. Differences in pCO_2 were associated with differences in the relative abundance of major bacterial phyla including Cyanobacteria, Bacteroidetes, Proteobacteria, Planctomycetes, and Verrucomicrobia. However, despite the differences in community composition, there is little sign of changes in the bacterial community that would be functionally significant in terms of nutrient cycling and ecosystem structure. As well as direct seawater pH effects, it is possible that increased growth of algae and decreased grazing contributed to the observed shift in bacterial community composition at high CO_2, as documented at several seep systems worldwide. Given the importance of biofilms to coastal ecology, changes in their composition due to globally rising pCO_2 levels requires further investigation to assess the implications for marine ecosystem function. However, the apparent lack of functional shifts in the biofilms despite the pH change may be a reassuring indicator of stability in shallow oceanic biofilm communities in the future.

Keywords: Bacteria, biodiversity, ocean acidification, rocky shore ecology.

Introduction

Bacteria dominate the abundance, diversity and metabolic activities of ocean ecosystems (Azam and Malfatti, 2007). On hard substrates they form attached biofilm communities where they interact in a matrix of extracellular polymeric substances (Decho, 2000; de Carvalho, 2018), which stick the cells to the substrata and protect them against environmental extremes. Marine biofilms rapidly colonise natural and artificial surfaces, and then facilitate the settlement of macroalgae and invertebrates (Lau et al., 2005; Qian et al., 2007). They cycle nutrients and organic matter, and can be a major source of primary productivity in the photic zone, providing an important food resource for higher trophic levels (Thompson et al., 2004; de Carvalho, 2018). Consequently, any changes in biofilm microbial diversity and abundance could have important implications for marine ecosystem structure and function (Weinbauer et al., 2011).

Anthropogenic CO_2 emissions are rapidly changing seawater chemistry, in a process called ocean acidification, and a major research priority is to find out how ecosystems will be affected by these changes (Riebesell and Gattuso, 2015). While marine organisms can tolerate large, rapid changes in carbonate chemistry in coastal regions (Wootton et al., 2008), the long-term effects of decreased mean pH and more frequent episodes of low carbonate saturation and high pCO_2 are poorly known. Areas that are naturally exposed to periods of lower pH, lower carbonate saturation and higher pCO_2 than those that occurred at pre-industrial levels of atmospheric CO_2 are helping to further our understanding of the ecosystem effects of ocean acidification (Hall-Spencer et al., 2008; Fabricius et al., 2011; Rastrick et al., 2018). This approach has shown that even in coastal ecosystems, where rapid changes in carbonate chemistry are normal, many calcified organisms are adversely affected when the frequency and duration of low carbonate saturation events increase. Concomitant pulses of high levels of dissolved inorganic carbon benefit certain primary producers, causing

shifts in their community composition (Porzio et al., 2011; Connell et al., 2013; Cornwall et al., 2017).

Ocean acidification may directly impact microbial diversity and composition, and indirectly affect microbes through links within food webs, for example with non-bacterial competitors and with grazers (Krause et al., 2012; Sunday et al., 2017). Despite the importance of microbial biofilms, few studies have investigated how ocean acidification might affect them (Liu et al., 2010; Kerfahi et al., 2014). Hypothetically, major biogeochemical processes driven by microbes may not be fundamentally different under future elevated pCO_2 conditions (Joint et al., 2011) since bacterial communities are able to reorganise themselves in response to environmental perturbations (Tolker-Nielsen and Molin, 2000). Aquarium-based experiments have been used to simulate these future conditions, but it is challenging to predict the responses of microbial communities based on these, as they do not incorporate feedbacks and the many indirect effects that might occur as a result of increased pCO_2 in natural systems – for example changes in geochemistry and organism interactions (Das and Mangwani, 2015). For this reason, carbon dioxide seep systems are now being used to investigate the long-term response of microbial communities to ocean acidification (Kerfahi et al., 2014; Morrow et al., 2014; Hassenrück et al., 2015, 2017; O'Brien et al., 2018).

Previous work on biofilm communities at carbon dioxide seeps suggests that their primary productivity will be enhanced by ocean acidification in the photic zone, at least where nutrient levels are sufficient (Lidbury et al., 2012; Johnson et al., 2015) and that there will be broad shifts in their community composition and diversity under increased pCO_2 (Kerfahi et al., 2014; Taylor et al., 2014). These biofilm community responses have also been shown in mesocosm-based studies using experimentally elevated pCO_2 (Witt et al., 2011) with no evidence of loss of biofilm function due to ocean acidification.

Natural biofilm communities on rocky shores are highly variable in terms of community structure and relative abundances, due to abiotic and biotic pressures (Williams et al., 2000), and rocky shore ecology is strongly influenced by shore type e.g. steep cliffs vs boulder fields, or hard vs soft rock substrata (Lewis, 1964). The response of microbial biofilms to the impacts of ocean acidification may differ, depending on habitat type, and may be caused directly by changes in carbonate chemistry, or indirectly due to effects on other organisms (Sunday et al., 2017).

In this study, our aim was to test whether the effects on coastal biofilm bacterial communities of increased seawater pCO_2 in the NW Pacific Ocean are similar to those observed in the Mediterranean Sea (Kerfahi et al., 2014). In Japan, Agostini *et al.* (2018) recently showed that in complex habitats, at increased levels of pCO_2 predominately calcareous organisms were replaced by simplified biofilm-dominated and algal turf-dominated habitats. We used the ocean acidification study system established by Agostini *et al.* (2018) to investigate the bacterial community structure of these biofilms on two types of low rocky shore habitat; cobble/boulder shores, and steep cliff faces. We examined four pCO_2 conditions, from pre-Industrial through to present day and projected future levels, to assess impacts of changing ocean chemistry on bacterial communities. We expected to find major changes in biofilm community composition, given the known sensitivity of bacterial communities to pH (e.g. Lauber et al., 2008; Tripathi et al., 2012), although this type of study has not previously been carried out in the Pacific Ocean.

Materials and methods

Study site

Sampling took place on the South coast of Shikine Island, East of the Izu peninsula, Japan in the North West Pacific Ocean (34° 32'N, 139° 20'E). The Izu archipelago is a chain

of islands approximately 150 km South of Tokyo (Fig. S1) situated at a subtropical-temperate biogeographic boundary due to the influence of the warm North flowing Kuroshio current (Agostini et al., 2018).

Sample collection and DNA extraction

Samples were collected from six sites along a pH gradient ranging from 7.2 to 8.3. At each site, six rock chips were randomly collected along a linear 1 m transect immediately above the low tide line. All samples had the same edaphic parameters, being on boulders (in boulder samples) or cliff faces (in cliff face samples) of the same basaltic lava, on flat faces at vertical westerly orientation and smooth texture. Rock chips were collected in June 2016 using a chisel and placed in individually labelled bags using clean forceps (Fig. S2) and immediately stored at -20˚C. DNA was extracted from each rockchip sample using the Power Soil DNA extraction kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol. The DNA samples were then sent to the Dalhousie University, Canada, for sequencing using an Illumina MiSeq platform.

Seawater chemistry

Geographical co-ordinates of each sample were taken using a GPS. A multi-sensor (U-5000G, Horiba Ltd, Kyoto Japan) was used to measure seawater pH, temperature, salinity and dissolved oxygen. To measure total alkalinity, 100 ml water samples were collected and then filtered at 0.45 μm with cellulose acetate filters (Dismic 045, Advantec Japan). Total alkalinity was measured via titration with HCl at 0.1 mol l^{-1} with a Metrohm titrator (785 DMP titrino) and total alkalinity was calculated by Gran plot from titration point with a pH between 4.0 and 3.0. Carbonate chemistry parameters: partial CO_2 pressure (pCO_2), Aragonite (Ar) and calcite (Ca) saturation state (Ωaragonite, Ωcalcite), and dissolved

149 inorganic carbon (DIC) were calculated using the CO_2SYS software package (Pierrot et al.,
150 2006) using temperature, salinity, pH (NBS scale) and total alkalinity (A_T) with the
151 disassociation constants from Mehrbach et al. (1973), as adjusted by Dickson and Millero
152 (1987), KSO_4 using Dickson (1990), and total borate concentrations from Uppström (1974).
153 The carbonate chemistry for the different sites is presented in Table 1.
154
155 **Sequence analysis**

156 The sequenced data generated from Miseq sequencing was processed using the
157 Mothur platform (Schloss et al., 2009). The sequences were aligned using Mothur (default
158 settings: kmer searching with 8mers and the Needleman–Wunsch pairwise alignment
159 method). Next, they were aligned against the EzTaxon-aligned reference (Chun et al., 2007),
160 and further filtered to remove gaps. Sequences were de-noised using the '*pre.cluster*'
161 command in Mothur implemented using a pseudo-single linkage pre-clustering algorithm
162 from Huse *et al.* (2010). Putative chimeric sequences were detected and removed via the
163 Chimera Uchime algorithm contained within Mothur (Edgar et al., 2011) in *de novo* mode,
164 which first splits sequences into groups and then checks each sequence within a group using
165 the more abundant groups as reference. The taxonomic classification was performed using
166 Mothur's version of the RDP Bayesian classifier, using EzTaxon-e database for each
167 sequence at 80% Naïve Bayesian bootstrap cut-off with 1000 iterations. The sequences used
168 in this study have been deposited in the NCBI Sequence Read Archive under accession
169 number SRP172073.
170
171 **Statistical analysis**

172 All samples were standardised by random subsampling to 13,882 reads per sample
173 using the sub.sample command (http://www.mothur.org/wiki/Sub.sample) in Mothur.

Richness, diversity indices, and rarefaction values were estimated using Mothur. Variation in the relative abundance of the bacterial phyla among the pH zones was tested using two-way ANOVA in R software package 2.14.2, with habitat type and pH level as fixed factors, after testing for normality. We used Tukey's Honest Significant Difference test for pairwise comparisons. We used the same procedure to test whether OTUs richness and diversity indices differed across different pH levels. OTU-based abundance data were first square root transformed to build the Bray-Curtis distance matrix using the vegdist function in the Vegan package of R (Oksanen et al., 2008). We performed a nonmetric multidimensional scaling (NMDS) plot using the metaMDS function in the vegan package of R. This used the Bray-Curtis distance matrix to assess patterns in bacterial species composition. A permutational multivariate analysis of variance (PERMANOVA) was performed with 999 permutations using the Adonis function in Vegan R package to test if bacterial community composition differed significantly by pH levels and habitat type.

The rank order nestedness relationship was also calculated on BINMATNEST (Rodríguez-Gironés and Santamaría, 2006) using default input parameters and null models to test whether the community assemblage in each treatment sample was a subset present in another sample. This approach calculates the p-value for rows and column totals and these were reordered following a packed matrix order from high-to-low nestedness as enumerated by (Dong et al., 2016).

Bacterial co-occurrence network analysis was conducted following methods given by (Faust and Raes, 2012; Lima-Mendez *et al.*, 2015). For each group, OTUs that appeared in three or less samples were removed. The relative abundance of OTUs was then used to construct networks using Spearman's correlation and Kullback-Leibler dissimilarity methods. Randomization generated permutation and bootstrap distributions, and *p* values were merged by Brown's method and multiple test correlation with Benjamini-Hochberg. Finally, edges

199 with p_{adj} above 0.05 were discarded.

200 The functional composition of the microbial community was analysed using PICRUSt
201 (Langille et al. 2013) on the OTUs generated from the 16S rRNA data. The PICRUSt
202 program requires a biom-formatted OTU table with OTUs assigned to a Greengenes OTU ID.
203 The 16S OTU table was normalized for copies of the 16S rRNA gene and exported as to
204 *.biom* format for analysis with the software package STAMP (Parks et al 2014). STAMP
205 includes statistical and visualization tools that were used to identify differences in functional
206 potential for the biofilm bacterial communities across different sites along the pH gradient. .
207 Multiple group comparisons of function abundance (KEGG module predictions) were
208 assessed using analysis of variance (ANOVA) followed by *Tukey–Kramer post hoc* tests
209 (confidence interval of 0.95) and reported with corrected *P* values (Storey's FDR multiple
210 test correction approach) in STAMP software (Parks et al 2014).

211

212 **Results**

213 We obtained 583,036 good quality sequences from 36 samples after rarefying to
214 13,882 reads per sample, which were classified into 10,296 operational taxonomic units
215 (OTUs) at 97% similarity level. Most samples showed no sign of reaching an asymptote in
216 OTU richness at the total number of reads available in the rarefaction analysis. This means
217 that more sequences would be required to assess the full taxonomic diversity of bacteria
218 within the biofilms (Fig. S3).

219 Alpha diversity of the bacterial community associated with biofilms across different
220 pH levels was calculated using OTU richness and diversity indices. Bacterial OTU richness
221 was significantly different across all intertidal sites at different pH levels, and highest number
222 of OTUs was observed at sites having the lowest pH (Fig. 1). The OTU richness differed
223 significantly by pH level ($F_{2, 30} = 27.05$, $P < 0.001$) and by the interaction between pH and

habitat type ($F_{2, 30}$ = 3.12, P = 0.05). Similarly, Shannon index differed significantly in relation to pH ($F_{2, 30}$ = 15.41, $P < 0.001$) and the interaction between pH and habitat type ($F_{2, 30}$ = 7.01, P = 0.003), with highest diversity found in sites having the lowest pH (Fig. 1). However, the community diversity did not differ between the boulder and cliff-face habitats (OTU richness: $F_{1, 30}$ = 0.83, P = 0.36; and Shannon index: $F_{1, 30}$ = 0.08, P = 0.77).

The most dominant bacterial sequences recovered in the present study belonged to *Cyanobacteria* and *Bacteroidetes* representing 36% and 34% of total reads, respectively, followed by *Proteobacteria* with 23% of total reads, and less than 3% for each of the following bacterial phyla: *Planctomycetes*, *Verrucomicrobia*, *Chloroflexi*, *Acidobacteria*, *Actinobacteria*, *Gemmatimonadetes* and *Chlorobi*. 3% of sequences remained unclassified.

The relative abundances of phyla in the epilithic bacterial community detected along the pH/CO_2 gradient off Shikine Island showed significant variations across different sampling sites except for *Chloroflexi* and *Chlorobi* (Table S1). The strongest shifts were observed in the most abundant bacterial phyla, with a significant decrease in the relative abundance of *Cyanobacteria* at reduced pH sites. The relative abundance of Bacteroidetes was decreased at medium and low pH, but then increased at very low pH sites. However, Proteobacteria exhibited an increase in relative abundance at low pH then a decrease at extreme low pH sites. The remaining phyla showed either no consistent patterns or no change in abundance across different sites (Fig. 2). The interaction between pH levels and habitat types had a more pronounced effect on bacterial community than only pH level or habitat type. *Cyanobacteria* were influenced by both factors, with lowest abundance was found in boulder zone and sites having lowest pH. Bacteroidetes were significantly affected only by pH with their lowest abundance observed at low pH sites for both boulder and cliff-faced sites. However, *Proteobacteria* were significantly affected only by habitat types showing a decrease in relative abundance at boulder sites. The interaction pH levels and habitat types

had a significant effect on most of detected phyla including *Verrucomicrobia*, *Acidobacteria*, *Actinobacteria* and *Gemmatimonadetes*. *Planctomycetes* were significantly affected by pH levels, habitat types and the interaction pH levels*habitat types (Fig. 2, Table S1).

More than 25 bacterial orders were detected in our sequences (Fig. 3a). *Sphingobacteriales* were the most abundant order, with a relative abundance of 13.8% of total reads but did not differ at both boulder and cliff-face sites along pH gradient. The relative abundance of almost all detected orders significantly differed in relation to at least one of the studied factors (pH level, habitat type, and interaction pH level*habitat type) (Table S2). The orders *Oscillatoriales*, *Cytophagales*, *Alteromonadales*, *Phycisphaerales*, *Kordiimonadales*, *Chromatiales* and *Balneola* were significantly affected by pH level, habitat type and the interaction of pH level and habitat type. However, *Flavobacteriales* (12.5% of total reads) the second common order significantly differed along pH level and the interaction pH level*habitat type and not with habitat type, with highest abundance found at ambient pH for boulder sites and low pH for cliff-face sites. The relative abundance of *Oscillatoriales* and *Vibrionales* was significantly lower at ambient pH for both boulder and cliff sites. Conversely, *Rhodospirillales* and *Sphingomonadales* were significantly greater at low pH for both habitat types. The remaining orders showed no consistent patterns (Fig. 3a).

At the family level, the biofilm bacterial communities at both boulder and cliff-face sites along pH gradient were dominated by *Saprospiraceae* (13.1% of total reads), *Flavobacteriaceae* (12% of total reads), *Prochlorotrichaceae* (7.9% of total reads), *Pleurocapsa* family (7.8% of total reads), *Vibrionaceae* (6.1% of total reads), *Rivulariaceae* (3.9% of total reads), etc. and had significant differences across pH level or/and habitat type except for *Saprospiraceae* ($P>0.05$) the most abundant family (Table S3). Most of the detected families showed no consistent pattern in response to pH and/or habitat type (Fig. 3b). For example, *Flavobacteriaceae* were higher at ambient pH in boulder sites and at low

pH in cliff-face sites. *Prochlorotrichaceae* were lower at ambient pH in both boulder and cliff sites. Only habitat type had a significant effect on *Vibrionaceae* and *Rivulariaceae* where *Vibrionaceae* represented a minor component of boulder sites (0.2% of total reads) and major component of cliff sites (14% of total reads), conversely *Rivulariacea*e were greater in boulder sites (6.5% of total reads) and lower in cliff sites (1.5% of total reads). *Pseudoalteromonadaceae* had also lower relative abundance in boulder sites (<0.01% of total reads) compared with cliff sites (7.5% of total reads). *Pseudoalteromonadaceae* were significantly influenced by pH, habitat type and the interaction pH level*Habitat type, with lower abundance observed at lower pH (Fig. 3b).

A non-metric multidimensional scaling (NMDS) using Bray-Curtis distance indicated that both pH levels and habitat type influenced bacterial community composition (Fig. 4). A permutational multivariate analysis of variance (PERMANOVA) results showed that bacterial community composition was not only affected by pH level ($F_{2,29}$ = 3.54, P = 0.001; Table 2) and habitat type ($F_{1,29}$ = 8.62, P = 0.001; Table 2), but also by the interaction between pH level and habitat type ($F_{2,29}$ = 3.65, P = 0.001; Table 2).

Nestedness analysis showed that bacterial communities followed a nested structure (p < 0.0001) across different pH levels. We generated a packed matrix order of all samples, in which the nestedness of each sample was categorised from high to low, and the lower ones are nested in the higher ones (Table S4). Samples having the lowest pH in boulder habitat and samples having ambient pH in cliff-face habitat had the lowest rank of nestedness compared to the other sites. Thus, the OTU composition of other sites could be a subset of the bacterial community in the 'Boulder-very low' and 'Cliff-ambient' sites.

Fig. 5 and Fig. 6 illustrated the interactions between taxa across different pH levels. Associations of bacterial taxa at very low pH sites outnumbered low and ambient pH sites, no matter the rock type. Positive edges were higher than negative edges, except for low pH cliff

and ambient boulder sites where the number of positive edges was equal with negative edges (Fig. 5). Furthermore, non-random edges distribution with regard to phylum was observed. We found that both intra-phylum and inter-phylum interaction edges of Bacteroidetes were the highest in all samples (Fig. 6).

The functional classification based on KEGG module predictions (Fig. S4) revealed that biofilm microbial communities show some a significant shift in most of the functions in Boulder and Cliff sites across different pH levels. Analysis of PICRUSt-predicted functional profiles for methane metabolic pathways suggest a decrease in gene abundance at lower pH for both boulder and cliff sites (Fig. 7a). Sulfur metabolism showed the opposite pattern with greater gene abundance at lower pH (Fig. 7b). The analysis of nitrogen metabolic pathway suggested no significant difference in gene abundance at both habitat types across different pH level (ANOVA, Tukey–Kramer, $P < 0.05$; Fig. 7c). Genes related to energy metabolic pathways increased at lower pH at both boulder and cliff sites (Fig. 7d). Carbon fixation potential in Prokaryotes showed an increase then a decrease in gene abundance related to carbon fixation in Prokaryotes at boulder sites along pH gradient. These genes decreased at medium pH then increased at low pH at cliff sites (Fig. 7e). The opposite pattern was observed for genes related to carbon fixation pathway in photosynthetic organisms (Fig. 7f).

Discussion

We found that bacterial community composition in biofilms on two types of rocky shore habitat was affected at CO_2 seeps (Heat map Fig). Pre-industrial, present day and projected future levels of seawater pCO_2 each had their own discrete communities of biofilm bacteria, raising the possibility that the transition from 300-400 ppm CO_2 in ocean seawater over the past 200 years has already affected biofilm communities worldwide. Biofilm growing at even higher than present-day levels of CO_2 increased in bacterial biodiversity.

This is very similar to the biofilm shifts recorded from present-day to high pCO_2 conditions in the Mediterranean Sea (Lidbury et al., 2012; Kerfahi et al., 2014) and in mesocosm conditions (Witt et al., 2011) and suggests a general response of biofilms to increasing CO_2.

Responses to ocean acidification conditions varied between bacterial taxa, some of these increasing whilst others decreased in relative abundance resulting in changes in species richness and diversity (Fig. 1, Fig. 2 and Fig. 3). These findings mirror those of Taylor *et al.* (2014) at CO_2 seeps in Italy, who found that shifts in bacterial biofilm communities involved a similar set of bacterial phyla to those recorded in our study (Fig. 2). Marine bacterioplankton communities also respond to changes in seawater CO_2 with changes in community composition, yet the functions of those communities remain resilient to acidification (Lindh et al., 2013; Lin et al., 2018), as predicted by Joint *et al.* (2011).

Holobiont communities can also be affected by ocean acidification conditions. Meron *et al.* (2012) reported shifts in coral bacterial community composition along a pH gradient of 8.1-7.3 caused by CO_2 seeps in Italy but found that many taxa were resilient. However, Webster *et al.* (2016) carried out an eight week-long mesocosm experiment and found that coral-associated bacterial taxa were tolerant of elevated pCO_2 (including *Proteobacteria*, *Bacteroidetes*, *Fusobacteria*, *Verrucomicrobia*, *Chloroflexi* and *Planctomycetes*) with no community shifts in these taxa between pH 8.1 (pCO_2 479–499 µatm) and pH 7.9 (pCO_2 738–835 µatm) although they acknowledged that a longer-term experiment might have revealed an effect. In contrast, shifts in hard substratum biofilm microbial community structure were recorded by Webster et al. (2013) who simulated the effects of ocean acidification by bubbling CO_2 into flow-through aquaria and recorded the appearance of new taxa within bacterial biofilm communities as the pH fell. This is similar to the responses we found in relation to pH treatments on Japanese rocky shores, suggesting that perhaps holobiont communities are under tighter control exerted by the host, compared to rocky

biofilm communities.

It is interesting that there are no major shifts in relative abundance functional groups, for example N fixers and photosynthesizers. Both taxonomic and functional analysis showed that major groups of bacteria (at family and genus levels) remained abundant at the higher pCO2 levels suggesting that the primary productivity and nutrient cycling of the system did not fundamentally changed, and that most essential biogeochemical functions were still present (Fig. 2) such as the nitrogen fixing activities *Cyanobacteria* and *Chloroflexi*.. It is also interesting to note that chemotrophs such as *Halothiobacillus*, *Guyparkeria*, *Thiobacillus*, *Sulfuritortus*, *Nitrosomonas*, *Gallionella* and *Ferriphaeselus* are absent even at the lowest pH treatments. This negative result simultaneously suggests that there may be little change in biogeochemical cycling as a result of such coastal biofilms, and confirms that the CO_2-rich vents are not contaminated with S-containing gases and are indeed pure CO_2 as was originally proposed based on gas and seawater chemistry measurements.

However, our conclusions on functional attributes of the biofilms are based only on taxonomic composition and relative abundances. It would be interesting to study the metagenome of these systems as a range of microbial functional processes are thought to be sensitive to ocean pH shifts (Das and Mangwani, 2015).

Network analysis revealed that sites having very low pH were more connected than low and ambient pH sites (Fig. 5 and Fig. 6). Network analysis which is based on statistically significant tests of correlation helps to illustrate interactions between taxa, and between particular taxa and particular gene functions (Barberán *et al.*, 2012; Faust and Raes, 2012; Mendes *et al.*, 2014). In networks based on correlation between bacterial OTUs, co-presence associations were stronger than mutual exclusion, implying that most interactions were positive at all studied pH levels, a pattern which is consistent with Lima-Mendez *et al.*, (2015). However, the nearly equal proportions of positive and negative edges demonstrate

374 that taxon-taxon interactions may be regulated by environmental factors. An intact species-
375 rich system would be expected to have greater network complexity due to stable and
376 predictable interactions (Wagg *et al.*, 2014). Our results showed that the sites with very low
377 seawater pH had more complex networks and higher diversity, which indicates that these
378 biofilm communities experienced greater environmental heterogeneity (Lin *et al.*, 2018).
379 *Bacteroidetes* exhibited the most intra-phylum and inter-phylum associations, suggesting that
380 *Bacteroidetes* play crucial roles in the biofilm communities we sampled.

381 Overall, we found that bacterial biofilms had considerable resilience to ocean water
382 acidification, retaining a similar high diversity and functional structure, and stronger network
383 complexity. This contrasts with the simplification of the taxonomic and functional diversity
384 system of larger organisms brought about by decreasing pH in these same coastal systems.
385 Agostini et al. (2018) showed that at the acidified sites on both of the habitat types we
386 studied, biofilm and turf algae predominated. The normal succession that occurs on rocky
387 shores had been truncated with simpler and less diverse macrobenthic communities, in line
388 with the findings of Kroeker et al. (2013) and Brown *et al.* (2018).

389 There are likely to be multiple mechanisms that drive changes in biofilm bacterial
390 composition along gradients of increasingly acidified conditions. These may include the
391 direct effects of low pH, or low carbonate, or high pCO_2 as well as the indirect effects that
392 occur due to changes in habitat and/or organism interactions. We consider it likely that the
393 bacterial community shifts we observed along pH gradients were party driven by changes in
394 the broader ecology of the rocky shore system food webs. At our high pCO_2 sites there was a
395 marked reduction in calcified grazers such as littorinid snails, limpets and chitons and an
396 increase in eukaryotic primary producers, such as diatoms (Agostini et al., 2018) all of which
397 would be expected to affect the thickness, extent and composition of biofilm communities.

398 To date, there had been no other studies on the effects of ocean acidification on

biofilms in Asia. Our comparison of marine biofilms along a natural pH gradient in Japan supports the view that while there may be pervasive effects of rising pCO_2 levels on marine bacterial biofilm communities, the functional effects may not be major. This warrants further investigation, given that acidification may disrupt biofilm settlement cues for commercially important shellfish, and may affect grazing habitat for a range of marine animals.

Our observations contrast with mounting evidence that increasing levels of seawater pCO_2 simplifies marine habitats, maintaining them in an early successional state (Kroeker et al., 2013; Brown et al., 2018). Agostini et al. (2018) showed that biodiverse rocky shore communities (including calcareous macroflora and fauna) were gradually replaced by simpler, less biodiverse communities that are dominated by non-calcified algae and biofilms in areas affected by periods of low carbonate saturation and high dissolved inorganic carbon. Here we showed that at the microbial level bacterial diversity and biofilm connectedness increased, and while communities shifted with unknown effects on biofilm function. Whilst the biofilms will still be fixing and cycling carbon and nutrients, it is possible that a range of other functions (e.g. provision of settlement cues) may be affected. Given the importance of biofilms to coastal ecology, changes in their composition due to globally rising pCO_2 levels requires further investigation to assess the implications for marine ecosystem function.

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Table legends

Table 1. Sea water chemistry across different sampling sites along pH gradient.

Table 2. Results of multivariate PERMANOVA showing significant differences in biofilm bacterial community composition between different sites along pH gradient.

Figure legends

Fig. 1. Biofilm bacterial alpha-diversity (mean ± SE) at different seawater pH levels in Shikine Island, Japan. Variation of OTUs richness in **(a)** boulder sites and **(b)** cliff sites. And variation of Shannon index **(c)** boulder sites and **(d)** cliff sites.

Fig. 2. Relative abundance of biofilm bacterial phyla detected along a pH gradient in Shikine Island, Japan.

Fig. 3. Relative abundance of biofilm bacterial taxa detected along a pH gradient in Shikine Island, Japan at **(a)** order level and **(b)** family level.

Fig. 4. NMDS ordination of total bacterial community composition among **(a)** different pH levels and **(b)** habitat types, based on Bray-Curtis distance.

Fig. 5. Frequency of co-occurrence patterns of sampled sites along a pH gradient in Shikine Island, Japan.

Fig. 6. Number of inter-Phylum and intra-Phylum co-presences and mutual exclusions of sampled sites (A: very low cliff site; B: low cliff site; C: ambient cliff site; D: very low boulder site; E: low boulder site; F: ambient boulder site).

Fig. 7. Box plot representing the relative abundance of the (a) Methane metabolism, (b) Sulphur metabolism, (c) Nitrogen metabolism, (d) Energy metabolism, (e) Carbon fixation in Prokaryotes and (f) Carbon fixation in photosynthetic organisms. The analysis was based on KEGG module

predictions using 16S data with the software PICRUSt. Results from multiple group comparisons in function abundance (ANOVA, Tukey–Kramer, $P < 0.05$) is reported as corrected *P*-value (Storey's FDR multiple test correction approach). The median value is shown as a line within the box and the mean value as a star. Error bars represent standard deviations.

Supplementary Online Material

Table S1. Results from two-way ANOVA showing the variation of the relative abundance of biofilm bacterial phyla across different sites.

Table S2. Results from two-way ANOVA showing the variation of the relative abundance of biofilm bacterial orders across different sites.

Table S3. Results from two-way ANOVA showing the variation of the relative abundance of biofilm bacterial families across different sites.

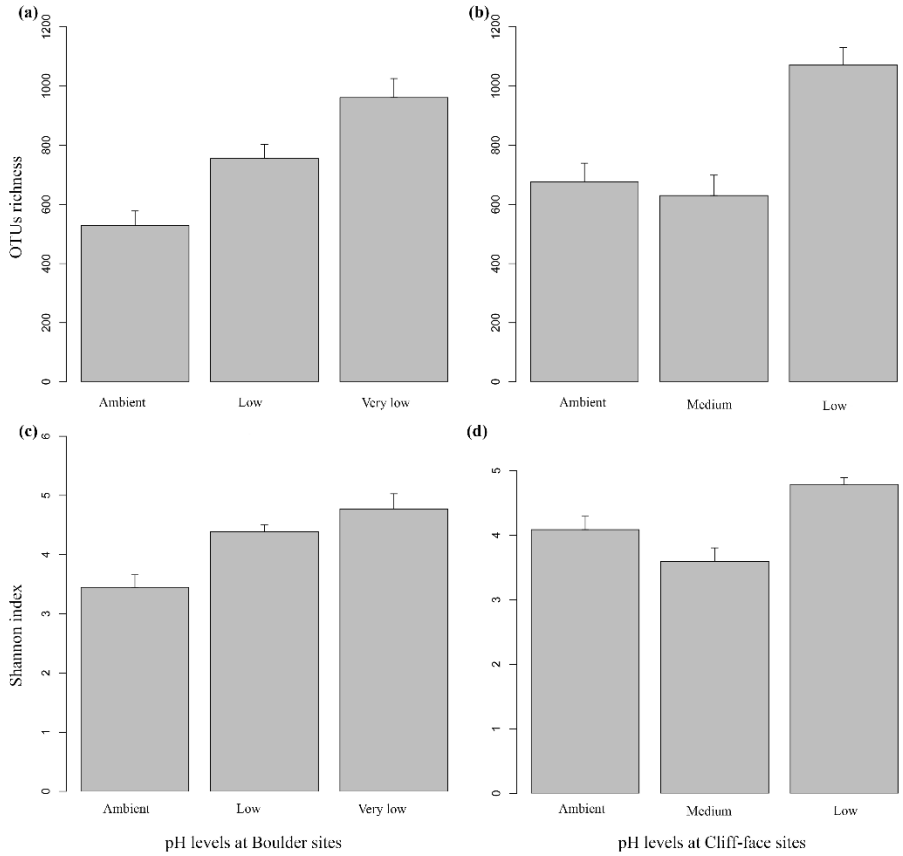
Table S4. Samples of "Boulder-Very low" and "Cliff-Ambient" sites formed the lowest nest while the other samples are basically a subset of these.

Fig. S1. Location of sample sites off Shikine Island, Japan.

Fig. S2. Rock biofilm sampling along a pH gradient off Shikine Island, Japan.

Fig. S3. Rarefaction curves comparing rock biofilm bacterial communities along a seawater pH gradient off Shikine Island, Japan.

Fig. S4. Predicted differences in biofilm bacterial functional categories (PICRUSt) across boulder and cliff sites off Shikine Island, Japan.



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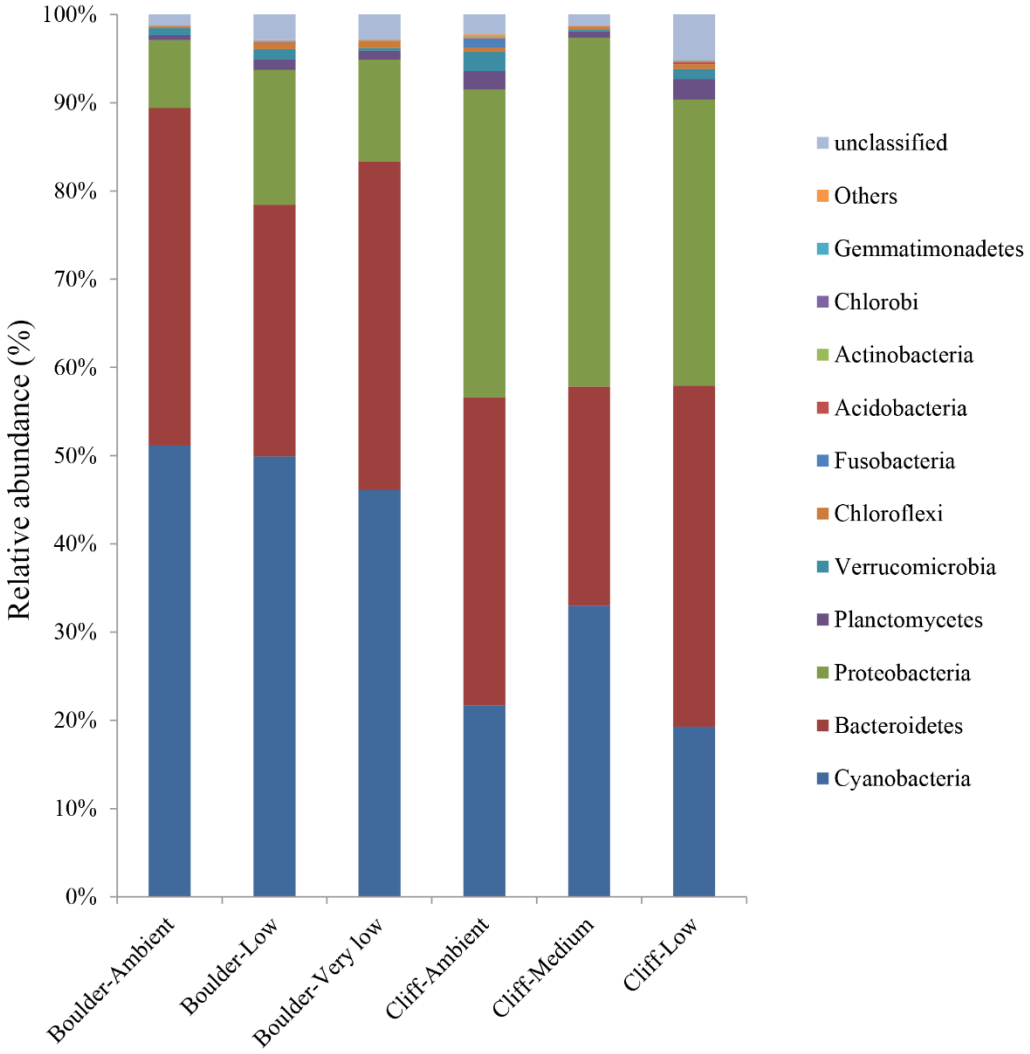
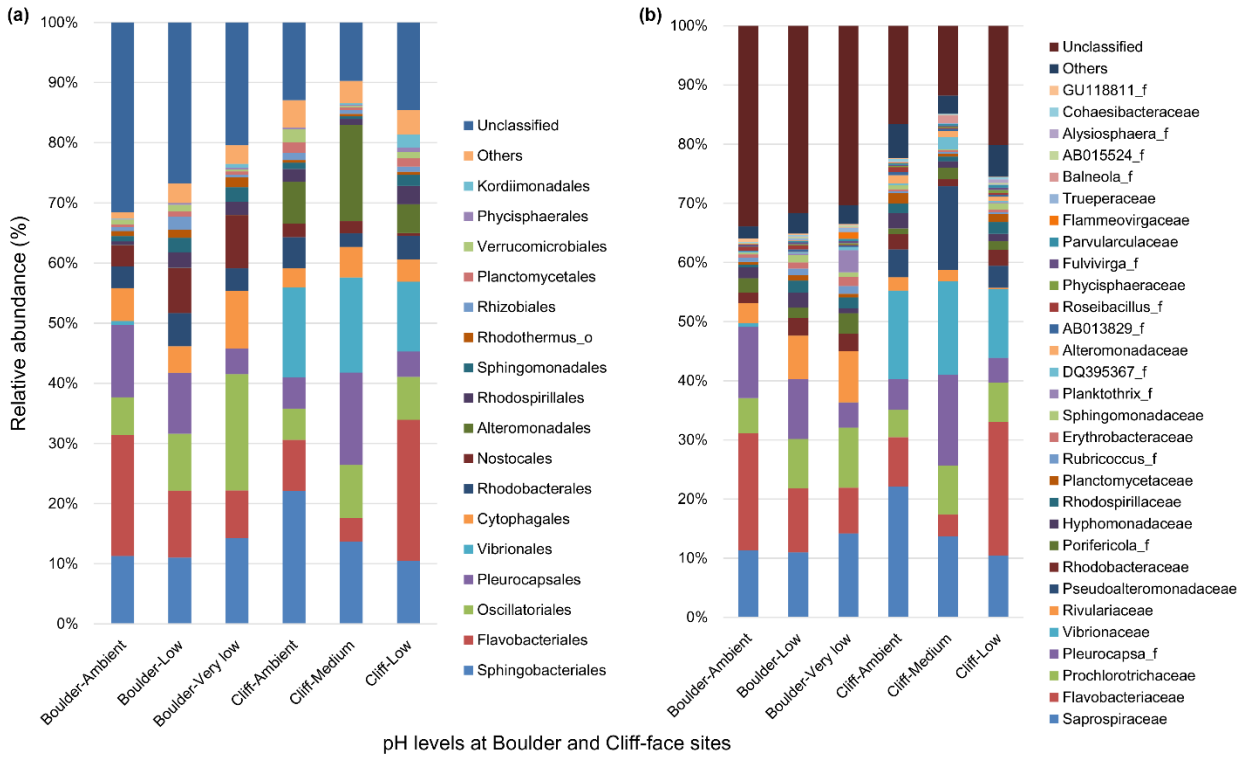


Fig. 2. Relative abundance of biofilm bacterial phyla detected along different sites in Shikine Island, Japan.



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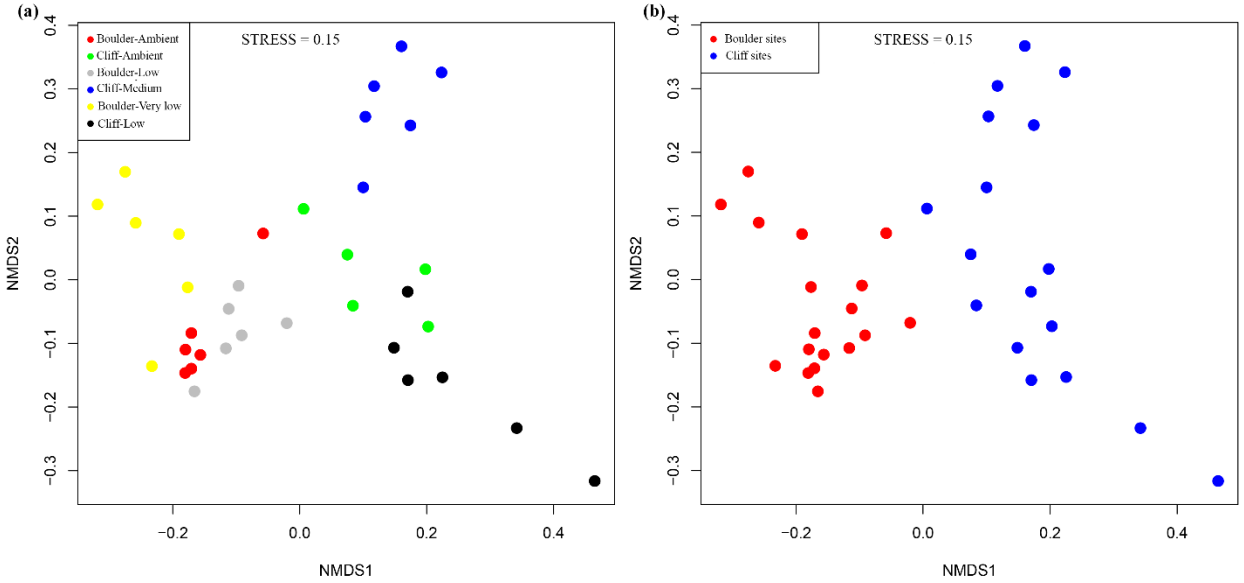

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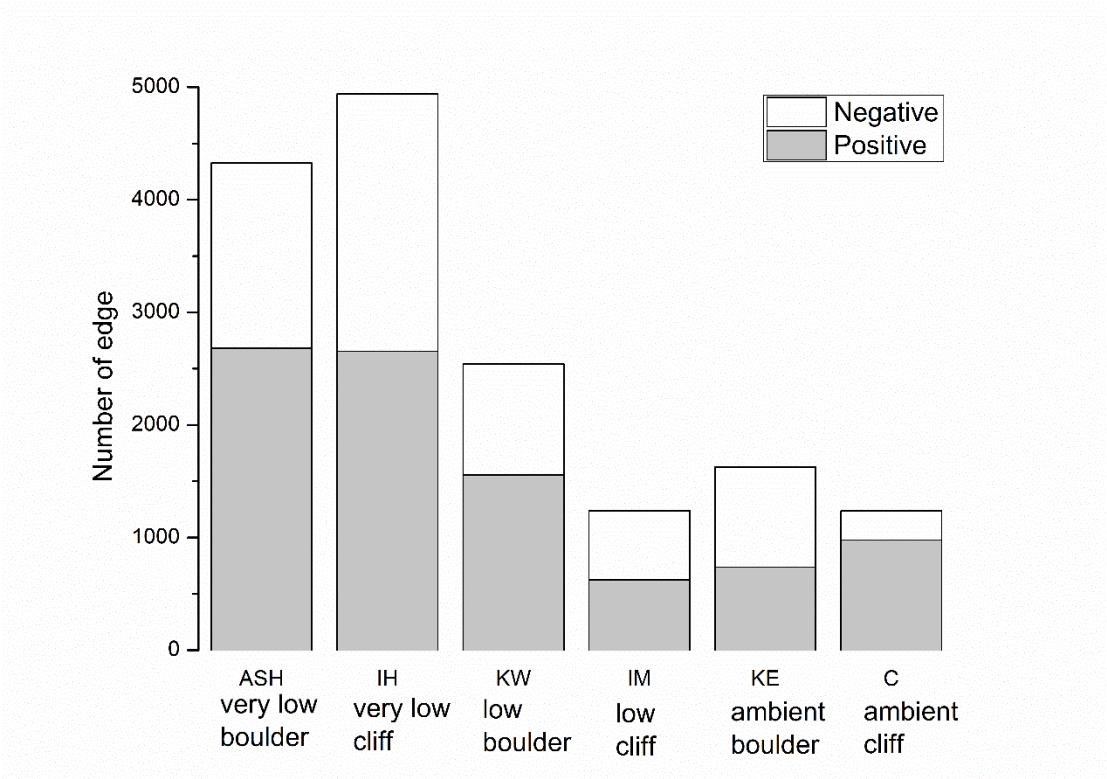
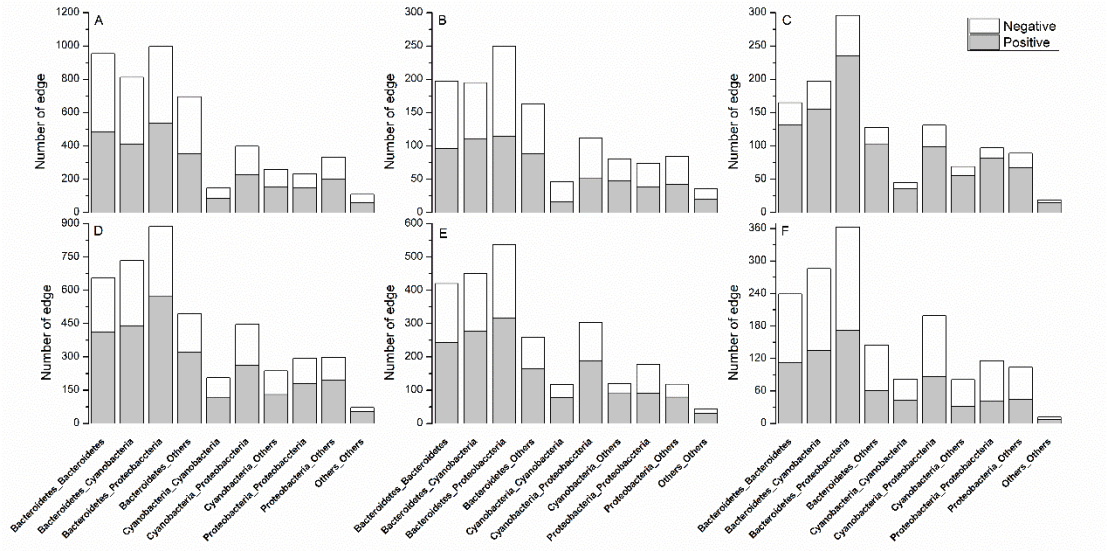


Fig. 5. Frequency of co-occurrence patterns of sampled stations.



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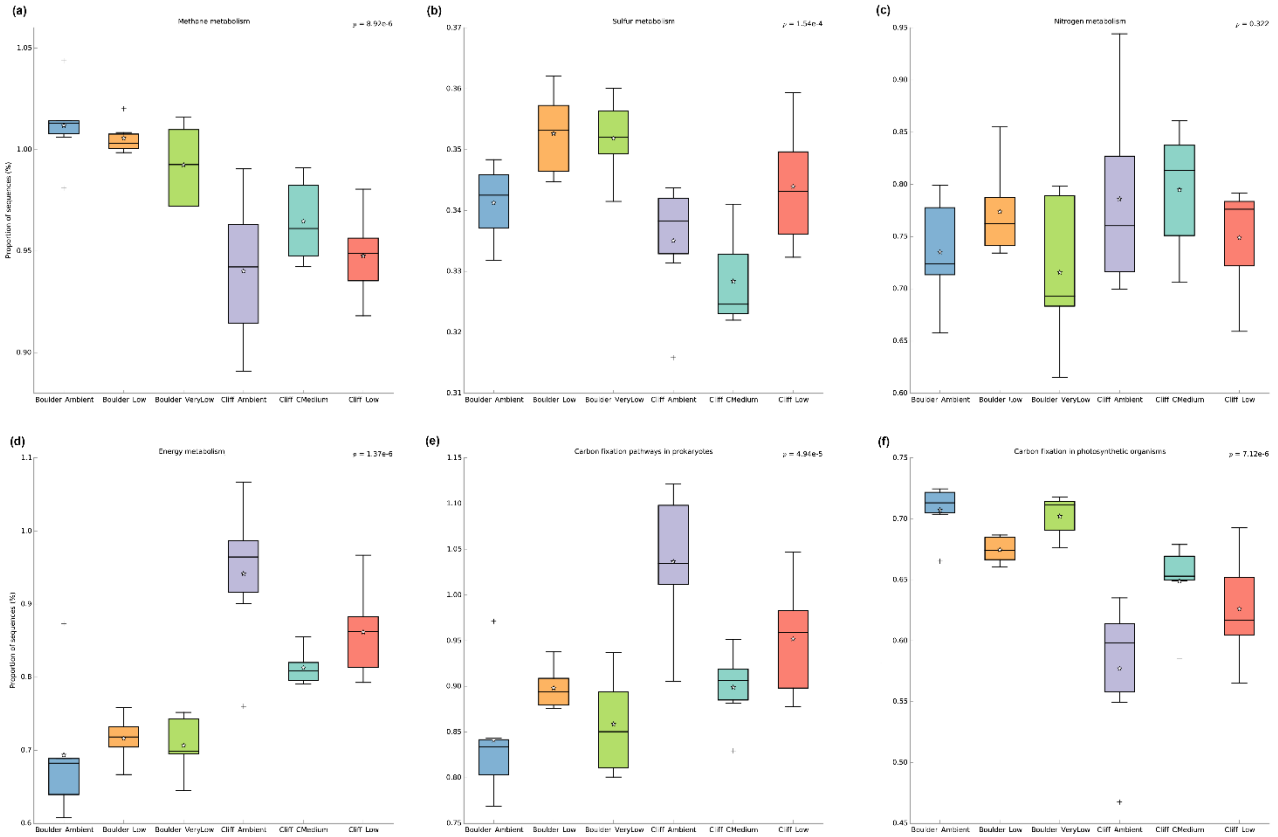

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Table 1. Sea water chemistry across different sampling sites along pH gradient.

Habitat type	pH zone	Salinity (psu)	Temp (°C)	A_T (µmol kg^{-1})	DIC (µmol kg^{-1})	pH_{NBS}	pCO_2 (matm)	HCO_3^- (µmol kg^{-1})	CO_3^{2-} (µmol kg^{-1})	Ω_{Ca}	Ω_{Ar}
Boulder	Ambient	34.0 ± 0.1	20.5 ± 0.7	2241.7 ± 12.5	1930.3 ± 20.6	8.28 ± 0.03	290.6 ± 25.1	1702.7 ± 33.1	218.3 ± 13.4	5.26 ± 0.32	3.42 ± 0.21
Boulder	Low	34.0 ± 0.2	20.4 ± 0.7	2201.4 ± 57.0	2141.5 ± 23.9	7.70 ± 0.07	1320.6 ± 225.4	2029.7 ± 27.9	69.4 ± 11.3	1.67 ± 0.27	1.09 ± 0.18
Boulder	Very low	34.1 ± 0.1	20.5 ± 0.5	2162.7 ± 31.7	2253.7 ± 37.6	7.24 ± 0.10	4003.9 ± 877	2100.2 ± 16.0	25.2 ± 6.5	0.61 ± 0.16	0.40 ± 0.10
Cliff	Ambient	34.0 ± 0.0	19.4 ± 0.5	2237.4 ± 1.0	1938.9 ± 36.3	8.27 ± 0.06	298.6 ± 51	1719.6 ± 58.1	209.4 ± 23.5	5.04 ± 0.57	3.27 ± 0.37
Cliff	Medium	34.0 ± 0.1	17.5 ± 1.1	2249.7 ± 2.1	2028.1 ± 35.9	8.15 ± 0.07	419.2 ± 82.3	1853.0 ± 55.1	160.5 ± 22.4	3.86 ± 0.54	2.49 ± 0.35
Cliff	Low	34.0 ± 0.0	18.5 ± 0.7	2281.7 ± 31.3	2186.8 ± 82.4	7.81 ± 0.22	1181.5 ± 672.4	2054.4 ± 103.6	92.3 ± 42.1	2.22 ± 1.01	1.44 ± 0.66

Supplementary Online Material

Responses of intertidal bacterial biofilm communities to increasing pCO_2 in a naturally acidified system

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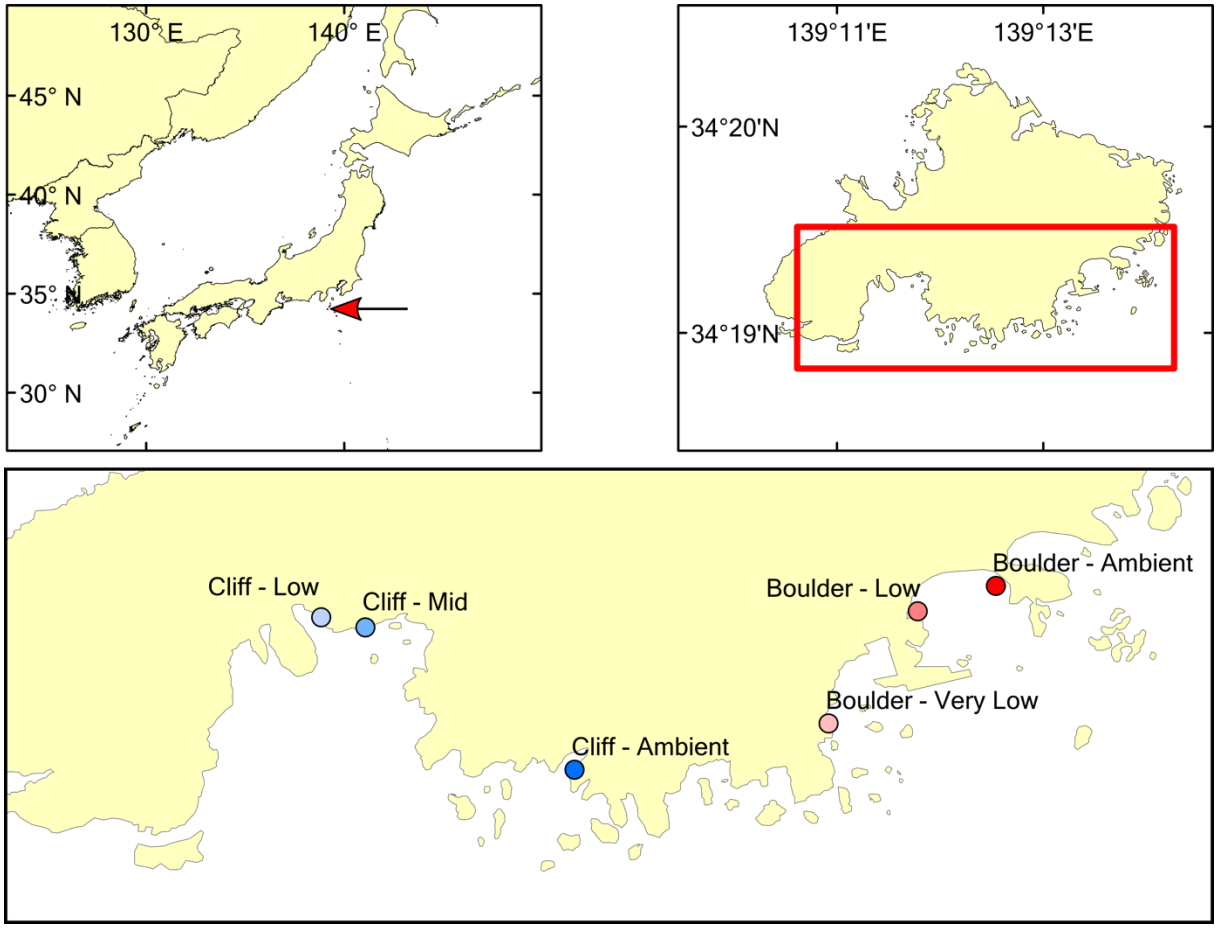

Fig. S1. Location of sample sites off Shikine Island, Japan.

Fig. S2. Rock biofilm sampling along a pH gradient off Shikine Island, Japan.

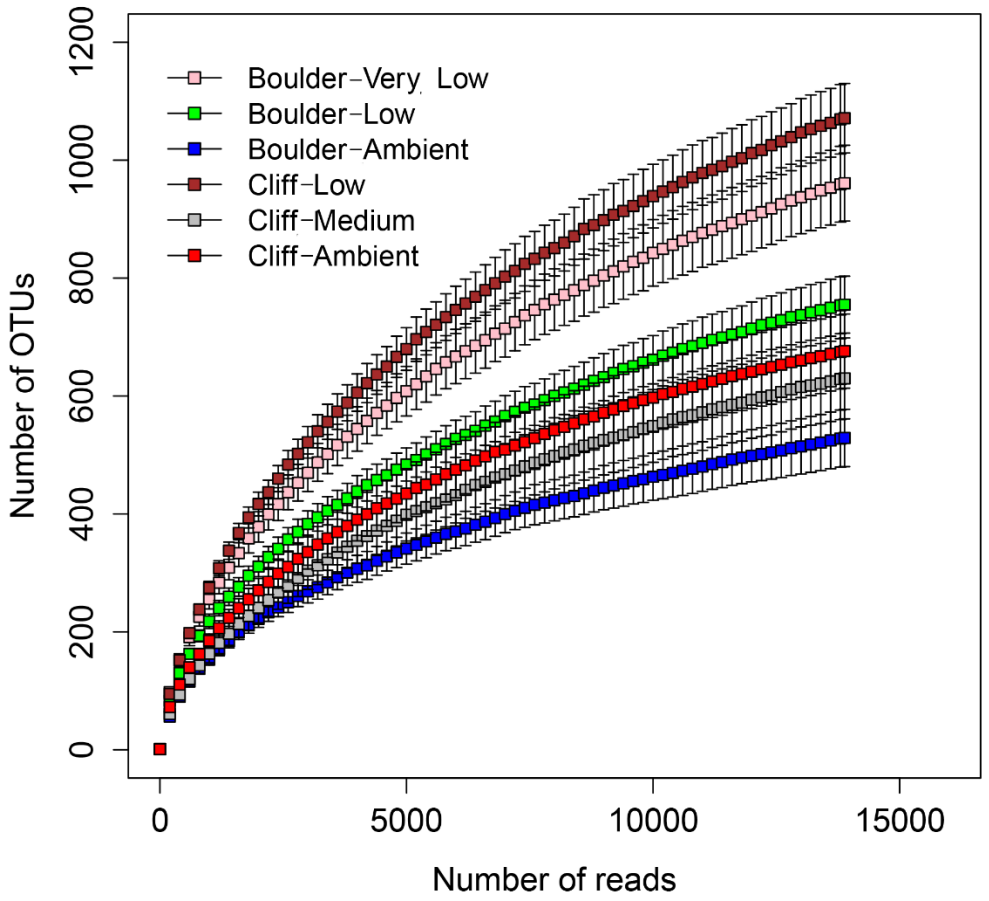

Fig. S3. Rarefaction curves comparing rock biofilm bacterial communities along a seawater pH gradient off Shikine Island, Japan.

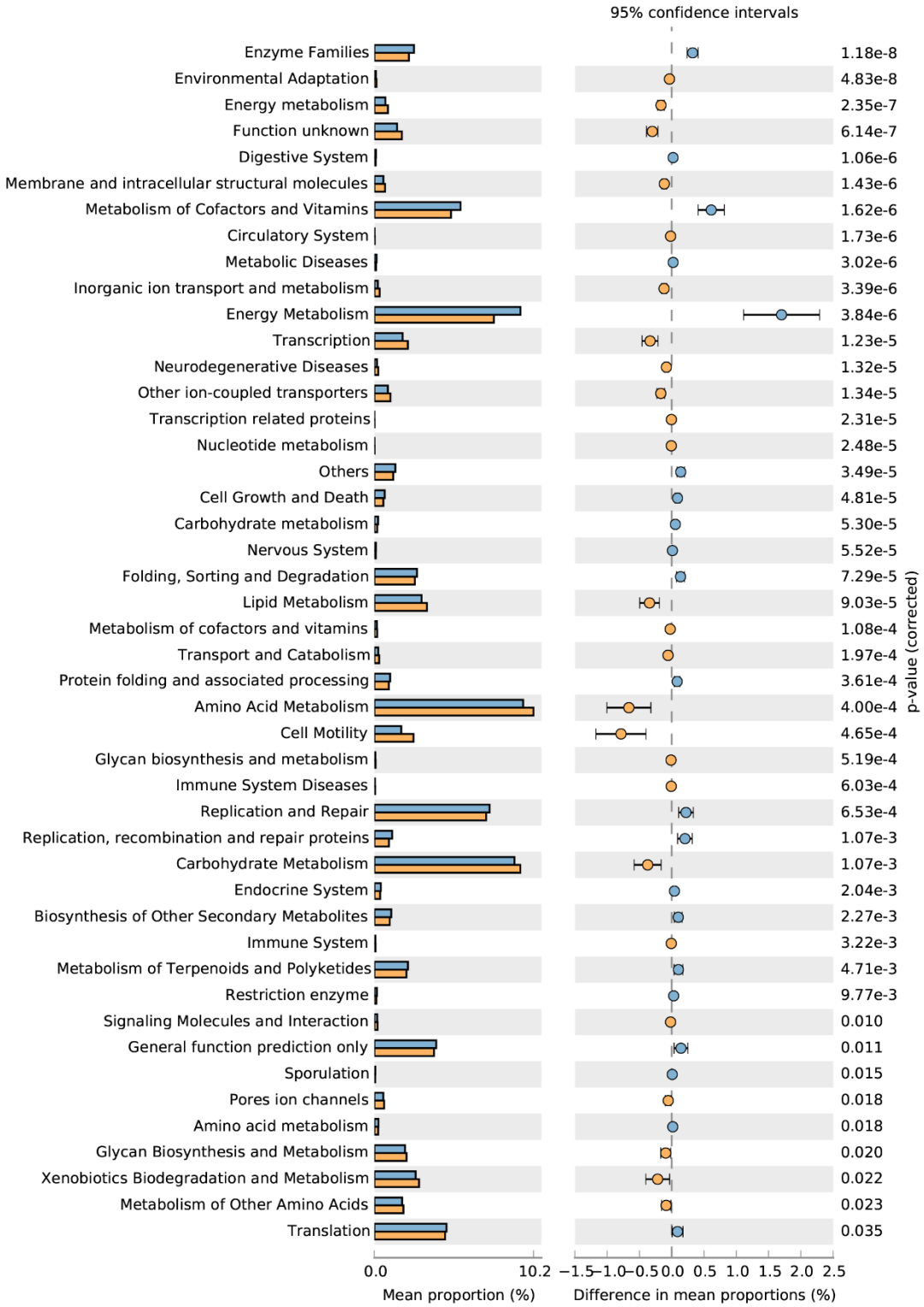

Fig. S4. Predicted differences in biofilm bacterial functional categories (PICRUSt) across boulder and cliff sites off Shikine Island, Japan.

Supplementary Online Material

Responses of intertidal bacterial biofilm communities to increasing pCO_2 in a naturally acidified system

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Table S1. Results from two-way ANOVA showing the variation of the relative abundance of biofilm bacterial phyla across different sites.

		df	Mean square	F model	P value
Cyanobacteria	**Habitat type**	**1**	**5279**	**67.76**	**<0.001**
	pH	2	234	3	0.064
	Habitat type * pH	2	133	1.7	0.19
Bacteroidetes	Habitat type	1	28.8	0.31	0.58
	pH	**2**	**451**	**4.85**	**0.01**
	Habitat type * pH	2	26.4	0.28	0.75
Proteobacteria	**Habitat type**	**1**	**5229**	**38.33**	**<0.001**
	pH	2	134	0.98	0.38
	Habitat type * pH	2	29	0.21	0.8
Planctomycetes	**Habitat type**	**1**	**5.81**	**12.48**	**0.001**
	pH	**2**	**1.52**	**3.27**	**0.05**
	Habitat type * pH	**2**	**3.67**	**7.9**	**0.001**
Verrucomicrobia	Habitat type	1	1.5	1.58	0.21
	pH	2	2.49	2.68	0.08
	Habitat type * pH	**2**	**4.38**	**4.61**	**0.01**
Chloroflexi	Habitat type	1	0.08	0.45	0.5
	pH	2	0.38	2.09	0.14
	Habitat type * pH	2	0.44	2.43	0.1
Fusobacteria	Habitat type	1	1.04	0.96	0.33
	pH	2	1.11	1.02	0.37
	Habitat type * pH	2	1.06	0.97	0.38
Acidobacteria	Habitat type	1	0.03	2.93	0.09
	pH	2	0.02	1.72	0.19
	Habitat type * pH	**2**	**0.05**	**3.71**	**0.03**
Actinobacteria	Habitat type	1	0.0004	0.07	0.79
	pH	2	0.009	1.47	0.24
	Habitat type * pH	**2**	**0.02**	**4.48**	**0.01**
Chlorobi	Habitat type	1	0.00005	0.02	0.8
	pH	2	0.006	3.02	0.06
	Habitat type * pH	2	0.001	0.52	0.59
Gemmatimonadetes	Habitat type	1	0.001	2.58	0.11
	pH	2	0.0009	1.7	0.19
	Habitat type * pH	**2**	**0.002**	**4.01**	**0.02**

Table S2. Results from two-way ANOVA showing the variation of the relative abundance of biofilm bacterial orders across different sites.

		df	Mean square	F value	P value
Sphingobacteriales	Habitat type	1	95.67	1.49	0.23
	pH	2	75.79	1.18	0.32
	Habitat type * pH	2	159.8	2.49	0.09
Flavobacteriales	Habitat type	1	11.9	0.31	0.57
	pH	**2**	**229**	**6.07**	**0.006**
	Habitat type * pH	**2**	**634.8**	**16.8**	**<0.001**
Oscillatoriales	**Habitat type**	**1**	**191.7**	**9.89**	**0.003**
	pH	**2**	**169.3**	**8.74**	**0.001**
	Habitat type * pH	**2**	**127.9**	**6.60**	**0.004**
Pleurocapsales	Habitat type	1	2.56	0.07	0.78
	pH	**2**	**217.5**	**6.19**	**0.005**
	Habitat type * pH	**2**	**109.4**	**3.11**	**0.05**
Vibrionales	**Habitat type**	**1**	**1734.4**	**10.25**	**0.003**
	pH	2	16.7	0.09	0.90
	Habitat type * pH	2	13.7	0.08	0.92
Cytophagales	**Habitat type**	**1**	**56.83**	**14.03**	**<0.001**
	pH	**2**	**18.16**	**4.48**	**0.01**
	Habitat type * pH	**2**	**32.84**	**8.10**	**0.001**
Rhodobacterales	Habitat type	1	2.10	0.61	0.43
	pH	2	1.11	0.32	0.72
	Habitat type * pH	**2**	**19.01**	**5.52**	**0.009**
Nostocales	**Habitat type**	**1**	**228.1**	**15.25**	**<0.001**
	pH	2	13.61	0.91	0.41
	Habitat type * pH	2	38.75	2.59	0.09
Alteromonadales	**Habitat type**	**1**	**760.9**	**32.70**	**<0.001**
	pH	**2**	**106.3**	**4.56**	**0.01**
	Habitat type * pH	**2**	**107**	**4.59**	**0.01**
Rhodospirillales	Habitat type	1	0.65	0.34	0.56
	pH	2	4.84	2.58	0.09
	Habitat type * pH	**2**	**8.12**	**4.32**	**0.02**
Rhodothermus_o	**Habitat type**	**1**	**6.38**	**32.18**	**<0.001**
	pH	2	0.59	2.98	0.06
	Habitat type * pH	2	0.44	2.22	0.12
Rhizobiales	Habitat type	1	0.25	0.96	0.33
	pH	**2**	**1.64**	**6.17**	**0.005**
	Habitat type * pH	**2**	**4.12**	**15.54**	**<0.001**

Planctomycetales	**Habitat type**	**1**	**2.80**	**9.29**	**0.004**
	pH	2	0.54	1.81	0.18
	Habitat type * pH	**2**	**2.27**	**7.53**	**0.002**
Verrucomicrobiales	Habitat type	1	1.45	1.51	0.22
	pH	2	2.67	2.79	0.077
	Habitat type * pH	**2**	**8.31**	**4.34**	**0.022**
Phycisphaerales	**Habitat type**	**1**	**0.25**	**5.86**	**0.02**
	pH	**2**	**0.56**	**12.91**	**<0.001**
	Habitat type * pH	**2**	**0.16**	**3.72**	**0.03**
Kordiimonadales	**Habitat type**	**1**	**3.02**	**4.21**	**0.04**
	pH	**2**	**6.74**	**9.41**	**<0.001**
	Habitat type * pH	**2**	**2.31**	**3.22**	**0.05**
Parvularculales	Habitat type	1	0.08	2.61	0.11
	pH	**2**	**0.20**	**6.22**	**0.005**
	Habitat type * pH	2	0.004	0.13	0.87
Balneola_o	**Habitat type**	**1**	**1.64**	**3.78**	**0.06**
	pH	**2**	**1.59**	**3.66**	**0.03**
	Habitat type * pH	**2**	**1.63**	**3.75**	**0.03**
Chromatiales	**Habitat type**	**1**	**0.23**	**32**	**<0.001**
	pH	**2**	**0.05**	**6.96**	**0.003**
	Habitat type * pH	**2**	**0.10**	**13.68**	**<0.001**
Myxococcales	Habitat type	1	0.002	0.52	0.47
	pH	**2**	**0.03**	**7.74**	**0.001**
	Habitat type * pH	2	0.01	2.71	0.08

Table S3. Results from two-way ANOVA showing the variation of the relative abundance of biofilm bacterial families across different sites.

		df	Mean square	F value	P value
Saprospiraceae	Habitat type	1	95.77	1.45	0.23
	pH	2	76.24	1.18	0.31
	Habitat type * pH	2	160	2.49	0.09
Flavobacteriaceae	Habitat type	1	13.8	0.37	0.54
	pH	**2**	**220.3**	**5.94**	**0.006**
	Habitat type * pH	**2**	**600.6**	**16.22**	**<0.001**
Pleurocapsa family	Habitat type	1	2.53	0.07	0.79
	pH	**2**	**217.3**	**6.19**	**0.005**
	Habitat type * pH	**2**	**109.4**	**3.11**	**0.05**
Vibrionaceae	**Habitat type**	**1**	**1734.4**	**10.25**	**0.003**
	pH	2	16.7	0.09	0.90
	Habitat type * pH	2	13.7	0.08	0.92
Rivulariaceae	**Habitat type**	**1**	**225.5**	**15.24**	**<0.001**
	pH	2	12.13	0.82	0.45
	Habitat type * pH	2	40.89	2.76	0.07
Pseudoalteromonadaceae	**Habitat type**	**1**	**508.4**	**22.64**	**<0.001**
	pH	**2**	**99.8**	**4.44**	**0.02**
	Habitat type * pH	**2**	**99.7**	**4.44**	**0.02**
Rhodobacteraceae	Habitat type	1	1.49	1.41	0.24
	pH	2	1.94	1.84	0.17
	Habitat type * pH	**2**	**5.21**	**4.94**	**0.01**
Porifericola family	**Habitat type**	**1**	**10.50**	**11.31**	**0.002**
	pH	2	2.01	2.17	0.13
	Habitat type * pH	**2**	**4.26**	**4.59**	**0.01**
Hyphomonadaceae	Habitat type	1	0.05	0.04	0.83
	pH	**2**	**4.24**	**3.75**	**0.03**
	Habitat type * pH	**2**	**4.42**	**3.91**	**0.03**
Planctomycetaceae	**Habitat type**	**1**	**2.80**	**9.29**	**0.004**
	pH	2	0.54	1.081	0.18
	Habitat type * pH	**2**	**2.27**	**7.53**	**0.002**
Rubricoccus family	**Habitat type**	**1**	**5.11**	**44.70**	**<0.001**
	pH	2	0.22	1.96	0.15
	Habitat type * pH	**2**	**0.36**	**3.14**	**0.05**
Erythrobacteraceae	**Habitat type**	**1**	**4**	**16.64**	**<0.001**
	pH	**2**	**0.94**	**3.94**	**0.03**
	Habitat type * pH	2	0.62	2.58	0.9

Sphingomonadaceae	Habitat type	1	0.28	1.41	0.24
	pH	**2**	**0.73**	**3.67**	**0.03**
	Habitat type * pH	**2**	**2.23**	**11.12**	**<0.001**
Planktothrix family	**Habitat type**	**1**	**14.42**	**15.06**	**<0.001**
	pH	**2**	**14.04**	**14.66**	**<0.001**
	Habitat type * pH	**2**	**12.01**	**12.54**	**<0.001**
Alteromonadaceae	**Habitat type**	**1**	**9.72**	**16.23**	**<0.001**
	pH	2	0.36	0.61	0.54
	Habitat type * pH	2	0.45	0.75	0.47
Phycisphaeraceae	**Habitat type**	**1**	**0.12**	**4**	**0.05**
	pH	**2**	**0.20**	**6.72**	**0.003**
	Habitat type * pH	2	0.03	1.30	0.28
Flammeovirgaceae	**Habitat type**	**1**	**1.29**	**6.75**	**0.01**
	pH	**2**	**1.09**	**5.68**	**0.008**
	Habitat type * pH	**2**	**1.22**	**6.40**	**0.004**
Alysiosphaera family	**Habitat type**	**1**	**0.32**	**6.45**	**0.01**
	pH	**2**	**0.28**	**5.61**	**0.008**
	Habitat type * pH	**2**	**0.39**	**7.9**	**0.001**
Cohaesibacteraceae	**Habitat type**	**1**	**0.15**	**8.2**	**0.007**
	pH	2	0.01	0.92	0.40
	Habitat type * pH	**2**	**0.14**	**7.37**	**0.002**

Table S4. Samples of "Boulder-Very low" and "Cliff-Ambient" sites formed the lowest nest while the other samples are basically a subset of these.

Group	Rank order of nestedness
Boulder-Very low 1	16
Boulder-Very low 2	15
Boulder-Very low 3	3
Boulder-Very low 4	5
Boulder-Very low 5	23
Boulder-Very low 6	13
Cliff-Ambient 1	14
Cliff-Ambient 2	17
Cliff-Ambient 3	1
Cliff-Ambient 4	2
Cliff-Ambient 5	6
Cliff-Low 1	12
Cliff-Low 2	31
Cliff-Low 3	33
Cliff-Low 4	10
Cliff-Low 5	18
Cliff-Low 6	4
Cliff-Medium 1	25
Cliff-Medium 2	11
Cliff-Medium 3	7
Cliff-Medium 4	35
Cliff-Medium 5	32
Cliff-Medium 6	22
Boulder-Ambient 1	30
Boulder-Ambient 2	34
Boulder-Ambient 3	19
Boulder-Ambient 4	8

Boulder-Ambient 5	27
Boulder-Ambient 6	24
Boulder-Low 1	20
Boulder-Low 2	29
Boulder-Low 3	9
Boulder-Low 4	21
Boulder-Low 5	28
Boulder-Low 6	26

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